SPECIFICATION

GLP-1 DERIVATIVE AND PREPARATION THEREOF ABSORBABLE VIA MUCOUS MEMBRANE

Technical Field

The present invention relates to novel human glucagon-like peptide-1 (GLP-1) derivatives absorbed highly via a mucous membrane in the oral cavity, lung, nose or intestines, production thereof and a method of using the same.

Background Art

GLP-1 (Glucagon-Like Peptide-1) is known as an incretin hormone which is secreted from digestive tracts upon ingestion of food to act on the pancreas and stimulate insulin secretion. As a hormone exhibiting a similar action, there is GIP (Gastric Inhibitory Polypeptide or Glucose-dependent Insulinotropic Polypeptide). This incretin effect is suggested to be absent or reduced in patients with type 2 diabetes, compared with healthy persons, and this is considered as one of causes high blood glucose. For example, it is reported that in patients with type 2 diabetes, blood GLP-1 level is lowered, while blood GIP level is normal. As a result of administering the incretin hormones to patients with type 2 diabetes, there is no difference upon the insulin secretion-stimulating activity of GLP-1 between the patients

and healthy persons, while the insulin secretion-stimulating activity of GIP is significantly lower in the patients than healthy persons. Accordingly, the response of the patients with diabetes to GLP-1 is maintained; thus, a GLP-1 preparation

5 compensating for its shortage can be expected to serve as a medicine for treatment of diabetes.

The action of GLP-1 on insulin secretion is characterized by glucose level dependent that GLP-1 does not stimulate insulin secretion in the blood glucose level of 110 mg/dL or less.. That

10 is, Administration of GLP-1 has clinicaladvantages that lower possibility of hypoglycemia, and suppress the excessive insulin secretion so that the exhaustion of the pancreas isis prevented. While A sulfonylurea, used mainly in treatment of type 2 diabetes, closes ATP-sensitive K^+ channels continuously to promote insulin

15 secretion it causes low blood glucose, exhaustion of the pancreas by excessive stimulation of β cells, and secondary failure in administration for a long period of time. Accordingly, the pharmacological characteristics of GLP-1 are very usefuland different from those of the conventional medicine for diabetes.

20 GLP-1 also have the following characteristics: suppression of glucagon secretion, delay of gastric emptying, suppression of stomach acid secretin, action on the brain to suppress appetite, promotion of insulin synthesis in pancreatic β cells and proliferation of pancreatic β cells. Therefore,

25 GLP-1 is considered not only effective for treatment of diabetes

by antagonizing the cause of high blood glucose such as hyperglucagonemia in type 2 diabetes, but also effective for treatment of obesity.

However, as GLP-1 is the polypeptide made up of 30 or 31
5   amino acids, it is digested upon oral ingestion and decomposed by digestive enzyme in the digestive tract, and is thus not absorbed. The administration thereof by intravenous injection or subcutaneous injection of GLP-1 is attempted at present. Further, it is known that GLP-1 undergoes
10   decomposition with dipeptidyl peptidase IV (DPPIV) present in blood or tissues so that the half-life thereof in the living body is as very short as 1 to 2 minutes, thus giving rise to an obstacle to clinical applications.

To solve these problems, some researches and developments
15   have been made. It is attempted to develop, for example, its derivative having an amino acid sequence substituted at position 8 which is hardly degradable to attain a longer half-life (Diabetologia 41: 271-278 (1998) and Biochem 40: 2860-2869 (2001)) and a sustained-release injection showing sustained
20   subcutaneous absorption. An injection of lizard-derived, synthetic Exendin-4 having a GLP-1 like agonistic activity and a long half-life in blood (Am J Physiol 281: E155-E161 (2001)) is also developed. However, an administration route other than via an injection is desired for wide application of GLP-1 as
25   a medicine for diabetes and in consideration of patients' burden

and convenience.

As an administration method not using an invasive means such as an injection or a preparation for oral administration, use of a preparation for absorption through mucous membranes in the lung, oral cavity, nasal cavity, vagina, eye, rectum and the like is available.. Buta peptide such as GLP-1 upon being administered alone, is poor in absorption via a mucous membrane. Accordingly, a polymer such as peptide is formulated together with an absorption promoter. To secure sustained absorption of chemicals, the preparation makes use of a water-soluble or water-swelling binder, and is formulated in the form of film adhering to the skin layer, buccal tablets, ointment or troche. Many absorption promoters or binders have been examined and found to be effective in facilitating administration of chemicals via mucous membranes. However, as the absorption rate, in terms of bioavailability, of GLP-1 in buccal tablets containing 400 µg GLP-1 through a mucous membrane in the human oral cavity reported by Gutniak et al. ("Diabetes Care" 20: 1874-1879 (1997)), even by using the prior art described above, is 7% of the absorption of its intravenous injection or 47% of the absorption of its subcutaneous injection, and its absorptivity is not sufficient.

Dipeptidyl peptidase IV is known as an enzyme degradating of GLP-1 , which is widely distributed not only in the kidney, liver, small intestine, salivary gland and various connecting tissues, but also in body fluid such as blood, urine and saliva

and in a mucous membrane in the nasal cavity, and also possibly in other mucous membrane tissues.

Disclosure of Invention

5   Absorption of GLP-1 via mucous membrane, as compared with absorption by an injection, is considerably ineffective due to low membrane permeability and degradation in absorption sites. Though the nasal administration of GLP-1 as such is not impossiblea very large dose of GLP-1 is necessary for achieving

10   a sufficient pharmacological effect. Accordingly, development of naturally occurring GLP-1 as a pharmaceutical preparation for nasal administration is unrealistic from the viewpoint of the production cost of GLP-1 peptide. For clinical application of GLP-1, it is necessary to develop GLP-1 derivatives whose

15   absorptivity via mucous membranes is comparative to that of an injection. Accordingly, the present inventors anticipated novel GLP-1 derivatives with improved absorptivity via mucous membranes, and made extensive studies to provide a preparation for mucous membrane administration as a substitute for an

20   injection.

       As a result, the inventors arrived at a novel idea that the absorption of GLP-1 via mucous membranes can be increased by adding positively charged arginine or lysine. In addition, they anticipated addition of several residues of arginine

25   and/lysine to the C-terminus thereof while the N-terminus

important for expressing the activity was kept intact, to obtain GLP-1 derivatives described below. For further increasing absorptivity via mucous membranes, a charge-regulated fat emulsion having a surface regulated to be negatively charged

5 was used to devise a GLP-1 preparation with significant improvement in mucous membrane absorption.

That is, the GLP-1 derivative of the present invention is a peptide comprising an amino acid sequence of GLP-1(7-35) having deletion, substitution and/or addition of one or a few

10 amino acids and having a GLP-1 activity with Waa-(Xaa)n-Yaa (in which Waa is Arg or Lys, Xaa is Arg or Lys, n is an integer of 0 to 14, and Yaa is Arg, $Arg-NH_2$, Lys, $Lys-NH_2$ or Hse) on its C-terminus of the peptide. As described above, a novel GLP-1 derivative showing high absorption via mucous membranes, that

15 is, high bioavailability via mucous membranes, can be provided by adding several residues of arginine and/or lysine to the C-terminus thereof.

To confer resistance to dipeptidyl peptidase IV, amino acid in position 8 of the GLP-1 derivative of the present invention

20 is preferably serine. Such peptide is represented by the general formula: $[Ser^8]$-GLP-1(7-35)-Waa-(Xaa)n-Yaa (in which Waa is Arg or Lys, Xaa is Arg or Lys, n is an integer of 0 to 14, and Yaa is Arg, $Arg-NH_2$, Lys, $Lys-NH_2$ or Hse).

The GLP-1 derivative of the present invention can be

25 endowed with trypsin resistance by substituting lysine at

position 26 with glutamine and lysine at position 34 with asparagine. Such peptide is represented by the general formula: [Gln^{26}, Asn^{34}]-GLP-1(7-35)-Waa-(Xaa)n-Yaa (in which Waa is Arg or Lys, Xaa is Arg or Lys, n is an integer of 0 to 14, and Yaa

5   is Arg, Arg-NH$_2$, Lys, Lys-NH$_2$ or Hse).

As a matter of course, these dipeptidyl peptidase IV-resistant or trypsin-resistant GLP-1 derivatives can also have deletion, substitution and/or addition of one or more amino acids in the amino acid sequence of GLP-1(7-35).

10   In the GLP-1 derivatives of the present invention, n is preferably an integer of 1 to 9, more preferably an integer of 3 to 5.

The most preferable peptide among the GLP-1 derivatives of the present invention is represented by the general formula:

15   [Ser^{8}, Gln^{26}, Asn^{34}]-GLP-1(7-35)-(Arg)n-Yaa (in which n is an integer of 4 to 6, and Yaa is Arg or Arg-NH$_2$).

The efficiency of absorption of the GLP-1 derivative was examined by administering the GLP-1 derivative of the present invention into mice via the nose, and then measuring its blood

20   glucose lowering activity and insulin secretion stimulating activity in the mice in a oral glucose tolerance test. As a result, the GLP-1 derivative showed a high blood glucose depressing action and insulin secretion promoting action, and the GLP-1 derivative of the invention in an amount of 1/10 relative

25   to naturally occurring GLP-1 showed a similar effect to that

of the naturally occurring GLP-1, and it is thus estimated that the absorption via the nasal mucous membrane is 10 times as high as that of the natural one.

For improving the efficiency of absorption of the GLP-1 derivative of the present invention, a charge-regulated fat emulsion described in JP-A 8-27018 was used to make a preparation. That is, the present invention also provides a GLP-1 preparation comprising the fat emulsion regulated to be negatively charged thereon and the GLP-1 derivative of the present invention.

The charge-regulated fat emulsion is a fat emulsion regulated to be negatively charged thereon, which is considered to absorb a peptide and protein thereby improving the stability of the peptide and protein against enzymes, simultaneously enhancing its pharmacological effect and extending duration. The GLP-1 derivative of the present invention, on one hand, has positively charged residues of arginine or lysine added thereto, and thus easily adheres to the charge-regulated fat emulsion. Accordingly, it is estimated that the mucous membrane absorption of the GLP-1 derivative of the present invention is increased when its preparation is produced by using the charge-regulated fat emulsion. When the efficiency of absorption of the GLP-1 derivative was actually examined by administering the preparation containing the GLP-1 derivative used in combination with the charge-regulated fat emulsion to mice in a glucose tolerant test and then measuring its blood glucose lowering

activity in the mice, the GLP-1 derivative of the present invention in an amount of 1/30 relative to the naturally occurring GLP-1 exhibited an effect similar to the natural one. That is, it is considered that the absorption of the GLP-1 derivative

5   of the present invention via the nasal mucous membrane, when used in combination with the charge-regulated fat emulsion, is 30 times as high as that of the naturally occurring GLP-1.

As described above, the GLP-1 derivative of the present invention is the most suitable peptide for making a preparation

10   having high absorptivity via mucous membranes, in particular, via the nasal mucous membrane. The GLP-1 derivative of the present invention, upon substitution at position 8 with serine, is made hardly degradable with dipeptidyl peptidase IV occurring in blood and tissues, thus giving a GLP-1 derivative having a

15   longer half-life in the living body. By conferring trypsin resistance as described above, the GLP-1 derivative can be protected against degradation with trypsin or trypsin-like enzymes and so on occurring in tissues, thus further improving bioavailability.

20   By combination with the charge-regulated fat emulsion, the GLP-1 derivative of the present invention can improve mucous membrane absorptivity to exhibit its effect in such a low dose as in an injection. That is, the present invention is to significantly improve the clinical applicability of a mucous

25   membrane absorption-type GLP-1 preparation, which can be easily

administered into patients without pain, to improve the quality of life of patients with diabetes and patients with obesity.

Best Mode for Carrying Out the Invention

5   Hereinafter, the present invention will be described in detail. GLP-1(7-35) is a peptide having the following sequence: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu -Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly (SEQ ID NO: 1). Herein, [Ser^8] means that amino acid at position

10   2 in this sequence, that is, Ala at position 8 is replaced by Ser, and [Ser^8] has the same meaning as 8S. Further, -NH$_2$ indicates that the sequence in question is amidated, and the GLP-1 derivative of the present invention can take a form amidated or not amidated at the C-terminus thereof.

15   The GLP-1 derivative of the present invention can be produced by chemical synthesis or genetic recombination techniques.

       The principle of chemical synthesis of polypeptides is known in the technical field of the present invention. For this

20   principle, reference can be made to the following general texts: Dugas H. and Penney C., "Bioorganic Chemistry" (1981) Springer-Verlag, New York, pp. 54-92; Merrifields JM, Chem. Soc., 85: 2149 (1962); and Stewart and Young, "Solid Phase Peptide Synthesis", items 24-66, Freeman (San Francisco, 1969). For

25   example, 430A peptide synthesizer (PE-Applied Biosystems Inc.,

850 Lincoln Center Drive, Foster City CA 94404) and a synthetic

cycle supplied by PE-Applied Biosystems can be used to synthesize

the peptide of the present invention by the solid phase method.

Boc amino acids and other reagents can be purchased from

5   PE-Applied Biosystems and other chemical suppliers.

Hereinafter, the method of producing the peptide of the

present invention by genetic recombination techniques will be

described in detail.

The DNA of GLP-1 is obtained by synthesis or by modifying

10   DNA encoding a larger naturally occurring glucagon. A DNA

sequence encoding a preproglucagon is shown by Lund et al. (Proc.

Natl. Acad. Sci. USA 79: 345-349 (1982)), and this naturally

occurring sequence can be changed to produce the compound of

the present invention. The method of constructing the synthetic

15   gene is known in the technical field of this invention, and

reference can be made to, for example, Brown et al.: "Methods

in Enzymology", 68: 109-151, Academic Press, NY. A DNA sequence

encoding the peptide of the present invention is designed on

the basis of the amino acid sequence of the peptide, and DNA

20   having the DNA sequence can be produced by a usual DNA synthesizer

such as Model 380A or 380B DNA synthesizer (PE-Applied Biosystems

Inc., 850 Lincoln Center Drive, Foster City CA 94404).

The DNA used in production of the GLP-1 derivative of the

present invention can be devised to increase its expression level

25   and accumulate the product stably in a host, devised to facilitate

purification after production, or devised to produce a fusion

protein from which the GLP-1 derivative can be easily cut off.

For example, such techniques can be exemplified by a technique

that involves linking a plurality of genes in tandem each encoding

5   the GLP-1 derivative of the present invention, to increase the

expression level, and a technique that involves linking the gene

to a gene of protein such as β-galactosidase, β-lactamase,

protein A or TrpE to produce the GLP-1 derivative as a fusion

protein.   In these cases, the GLP-1 derivative can be obtained

10   as its sole peptide after production by previously inserting

a gene corresponding to an amino acid methionine into between

the respective genes and treating the product with cyan bromide.

In this case, the C-terminus of this product is Hse (homoserine).

Some GLP-1 derivatives of the present invention have arginine

15   at the C-terminus only, and can be enzymatically treated with

arginyl endopeptidase to give the GLP-1 derivative as its sole

peptide.

For efficiently expressing the GLP-1 derivative peptide,

its synthetic DNA having a predetermined sequence is inserted

20   into a suitable recombinant DNA expression vector by suitable

restriction endonuclease.   Generally, reference can be made to

a literature of Maniatis et al. ("Molecular Cloning", A

Laboratory Manual, Vols. 1-3 (1989), Cold Springs Harbor

Laboratory Press, NY).   To achieve efficient transcription of

25   the synthetic gene, the gene is operatively linked to a

promoter/operator region. The synthetic gene promoter/operator region is arranged in the same sequence orientation with respect to the ATG initiation codon of the synthetic gene. Various expression vectors usable in

5   transformation of procaryotic cells and eucaryotic cells are known, and reference can be made to "The Promega Biological Research Products and Catalogue" and "The Stratagene Cloning Systems Catalogue".

      After construction of the expression vector for the GLP-1

10   derivative peptide, the vector is used to transform suitable host cells. As the host cells, either eucaryotic or procaryotic cells can be used. Techniques for transforming the cells are known in this field, and can be found in general literatures such as the above literature of Maniatis et al. Generally, the

15   procaryotic host cells produce a protein in a higher ratio and can be cultured more easily. A protein expressed in a high-level microbial expression system is uniquely aggregated to form particles or inclusion bodies containing the protein expressed in excess at high level. Such typically aggregated protein is

20   solubilized by techniques known in this field, denatured and folded again. For such techniques, reference can be made to "Protein Folding", Kreuger et al. (1990), pp. 136-142, ed. Gierasch and King, American Association for Advancement of Science Publication.

25   The GLP-1 derivative of the present invention can be

combined with a pharmaceutically acceptable carrier, diluent, excipient or absorption promoter to prepare a pharmaceutical composition. The absorption promoter includes, for example, a chelating agent (for example, EDTA, citric acid, salicylate),

5 a surfactant (for example, sodium dodecylsulfate (SDS)), a non-surfactant (for example, unsaturated cyclic urea) and bile acid salts (for example, sodium deoxycholate, sodium taurocholate). Such pharmaceutical compositions can be produced by methods known in the field of pharmaceutical

10 manufacturing. These pharmaceutical preparations are suitable for administration via a mucous membrane in the nasal cavity or the like, and can be used alone or in combination with other therapeutic agents. The GLP-1 derivative of the present invention can also be formed into injections, oral preparations

15 and the like other than the preparation for mucous membrane administration.

Using methods known in the technical field of the present invention, the composition of the present invention can be formed into a preparation to provide continuous or sustained release

20 of the active ingredient immediately after administration into patients. For example, suitable macromolecules (for example, polyester, polyamino acid, polyvinyl pyrrolidone, ethylene vinyl acetate, methyl cellulose, carboxymethyl cellulose and protamine sulfate), or a polymer substance such as polyester,

25 polyamino acid, hydrogel, poly(lactic acid) or ethyl vinyl

acetate copolymer can be used to form a complex of the peptide of the present invention or to absorb the peptide of the present invention, in order to produce a preparation showing controlled release.   Instead of mixing the peptide with particles of these

5   polymers, the peptide of the present invention can be encapsulated in microcapsules produced by coacervation techniques or interfacial polymerization, microcapsules including hydroxymethyl cellulose or gelatin, in colloidal drug delivery system (for example, liposomes, albumin, microspheres,

10   microemulsion, nano-particles and nano-capsules) or in microemulsion.

In the present invention, a preparation with further improvement in absorption of the peptide of the present invention via mucous membranes can be produced by absorbing the peptide

15   of the present invention onto a charge-regulated fat emulsion prepared according to JP-A 8-27018. As the charge regulator, at least one substance selected from various acidic phospholipids and salts thereof, various fatty acids and salts thereof, bile acids and salts thereof is used. The acidic phospholipids and

20   salts thereof include, but are not limited to, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, phosphatidic acid and salts thereof. The fatty acids and salts thereof are not particularly limited either, but are preferably C6 or more fatty acids and salts thereof. The bile acids and

25   salts thereof include, but are not limited to, dehydrocholic

acid, deoxycholic acid, taurocholic acid and salts thereof. By selecting the charge regulator and establishing the concentration of the charge-regulated fatty emulsion, the pharmaceutical composition of the present invention suitable
5 for administration site can be prepared.

The GLP-1 derivative of the present invention is effective against various diseases against which a GLP-1 preparation is effective. That is, the GLP-1 derivative of the present invention can be used to treat non-insulin dependent mellitus
10 diabetes, insulin-dependent mellitus diabetes, obesity and/or suppression of appetite.

The dose of the GLP-1 derivative of the present invention is determined desirably by those skilled in the art, depending on the respective patients with various diseases. Generally
15 speaking, however, the amount of the GLP-1 derivative to be administered once is considered to be in the range 1 µg/kg to 1 mg/kg, preferably 10 µg/kg to 100 µg/kg. Just before a meal, the GLP-1 derivative is administered once to thrice or more times every day.

20 Hereinafter, the present invention is described in more detail by reference to Examples and Test Examples. However, these examples are not intended to limit the scope of the present invention.

Production Example: Synthesis of GLP-1 derivatives

25 Synthesis of GLP-1 derivative was conducted by solid phase

synthesis with Model 430A Peptide Synthesizer (PE-Applied

Biosystems, Foster City, CA), and the products were purified

by HPLC, and the synthesized products were confirmed by mass

spectrometry. The purity of a majority of samples was 95% or

5   more, and such samples were used in in vitro and in vivo tests.

Hereinafter, the synthesized compounds are shown. The

sequence of GLP-1(7-36) is

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu

-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Ar

10 g (SEQ ID NO: 2) (that is, GLP-1(7-36) is the same as

GLP-1(7-35)-Arg). For example, GLP-1(7-36)+Arg-NH_2 is a

compound comprising naturally occurring G-GLP-1(7-36) having

one amidated Arg residue added to the C-terminus thereof.

Moreover, [Ser[8]]-GLP-1(7-35) is a compound wherein Ala at

15 position 2 (corresponding to position 8) was replaced by Ser

and the last (corresponding to position 36) Arg was deleted.

Comparative Production Example 1: GLP-1(7-36)-NH_2, which is the

naturally occurring GLP-1

Comparative Production Example 2: [Ser[8]]-GLP-1(7-35)-Arg-NH_2

20 (SEQ ID NO: 3), which is abbreviated to 8S-GLP-1

Production Example 1: GLP-1(7-36)+Arg-NH_2 (SEQ ID NO: 4), which

is abbreviated to GLP-1+1R

Production Example 2: GLP-1(7-36)+Arg-Arg-NH_2 (SEQ ID NO: 5),

which is abbreviated to GLP-1+2R

25 Production Example 3: [Ser[8]]-GLP-1(7-35)-Arg-Arg-Arg-NH_2 (SEQ

ID NO: 6), which is abbreviated to 8S-GLP-1+2R

Production Example 4: [Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-NH$_2$

(SEQ ID NO: 7), which is abbreviated to 8S-GLP-1+3R

Production Example 5:

5   [Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-NH$_2$ (SEQ ID NO: 8),

which is abbreviated to 8S-GLP-1+4R

Production Example 6:

[Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-Arg-NH$_2$ (SEQ ID NO: 9),

which is abbreviated to 8S-GLP-1+5R

10   Production Example 7:

[Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH$_2$ (SEQ ID

NO: 10), which is abbreviated to 8S-GLP-1+6R

Production Example 8:

[Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH$_2$

15   (SEQ ID NO: 11), which is abbreviated to 8S-GLP-1+8R

Production Example 9: [Ser^8]-GLP-1(7-35)-Lys-Arg-NH$_2$ (SEQ ID

NO: 12), which is abbreviated to 8S, des36R-GLP-1+1KR

Production Example 10: [Ser^8]-GLP-1(7-35)-Lys-Lys-Arg-NH$_2$ (SEQ

ID NO: 13), which is abbreviated to 8S, des36R-GLP-1+2KR

20   Production Example 11: [Ser^8]-GLP-1(7-35)-Lys-Lys-Lys-Arg-NH$_2$

(SEQ ID NO: 14), which is abbreviated to 8S, des36R-GLP-1+3KR

Production Example 12:

[Ser^8]-GLP-1(7-35)-Lys-Lys-Lys-Lys-Lys-Arg-NH$_2$ (SEQ ID NO: 15),

which is abbreviated to 8S, des36R-GLP-1+5KR

25   Production Example 13:

[Ser^8]-GLP-1(7-35)-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Arg-NH$_2$ (SEQ ID NO: 16), which is abbreviated to 8S, des36R-GLP-1+7KR

Production Example 14:

[Ser^8]-GLP-1(7-35)-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-

5　Arg-NH$_2$ (SEQ ID NO: 17), which is abbreviated to 8S, des36R-GLP-1+10KR

Production Example 15: [Ser^8]-GLP-1(7-35)-Arg-Lys-Lys-NH$_2$ (SEQ ID NO: 18), which is abbreviated to 8S-GLP-1+2K

Reference Production Example 1: [Ser^8, Gln^{26},

10　Asn^{34}]-GLP-1(7-35)-Arg (SEQ ID NO: 19), which is abbreviated to 8S26Q34N-GLP-1

Reference Production Example 2: [Gln^{26},

Asn^{34}]-GLP-1(7-35)-Arg-NH$_2$ (SEQ ID NO: 20), which is abbreviated to 26Q34N-GLP-1

15　　　　Besides these production examples, GLP-1 derivatives such as Production Example 16, [Ser^8, Gln^{26},

Asn^{34}]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-NH$_2$ (SEQ ID NO: 21) (abbreviated to 8S26Q34N-GLP-1+4R); Production Example 17, [Ser^8, Gln^{26},

20　Asn^{34}]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH$_2$ (SEQ ID NO: 22) (abbreviated to 8S26Q34N-GLP-1+6R); and Production Example 18, [Ser^8, Gln^{26},

Asn^{34}]-GLP-1(7-35)-Lys-Lys-Lys-Lys-Lys-Arg-NH$_2$ (SEQ ID NO: 23) (abbreviated to 8S26Q34N, des36R-GLP-1+5KR) are considered

25　preferable.

With respect to Production Examples 1 to 15 and Production Example 16 to 18, the C-terminus is amidated (-NH$_2$), but such derivatives can be non-amidated (-OH) derivatives. For example, the non-amidated (-OH) derivative in Production Example 5 can

5 be made the derivative in Production Example 19, that is, [Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Arg (SEQ ID NO: 24) (abbreviated to 8S-GLP-1+4R). The C-terminus can be Hse. Such peptide can be exemplified by the derivative in Production Example 20, that is, [Ser^8]-GLP-1(7-35)-Arg-Arg-Arg-Arg-Hse

10 (SEQ ID NO: 25) (abbreviated to 8S-GLP-1+3RHse).

Test Example 1: Cyclic AMP-producing activity of GLP-1
derivatives

An expression vector of GLP-1 receptor was constructed on the basis of a published DNA sequence of human GLP-1 receptor

15 (Graziano et al., Biochem Biophys Res Com 196: 141-146 (1993)). Chinese hamster ovary CHO-K1 cells were transformed with the vector to give recombinant CHO-K1 cells expressing human GLP-1 receptor.

The human GLP-1 receptor-expressing cells were put at a

20 density of 1 × 10^4 cells/ml/well on a 24-well plate. After 3 days, the cells were used in assay and incubated together with each GLP-1 derivative at 37°C for 30 minutes in a buffer (PBS, 5.6 mM glucose, 1 mM isobutyl methyl xanthine, 20 μM Ro20-1724, 0.5% BSA, pH 7.4). 10 μl of 5 N hydrochloric acid was added

25 to terminate the incubation.

Cyclic AMP products formed in the cells by the reaction of various GLP-1 derivatives with the GLP-1 receptor were measured by enzyme immunoassays with cAMP-Screen™ system (Applied Biosystems). The cyclic AMP-producing activities of 5 the various GLP-1 derivatives, in terms of relative activity to that (= 100%) of naturally occurring GLP-1, are shown in Table 1.

Table 1

Cyclic AMP-producing activities of various GLP-1 derivatives, in terms of relative activity to that (= 100%) of naturally occurring GLP-1, in receptor-expressing cells

GLP-1 derivatives	Concentration of GLP-1 derivative (M)		
	1×10^{-11}	1×10^{-10}	1×10^{-9}
GLP-1	100.0	100.0	100.0
GLP-1+1R	7.4	100.6	78.1
GLP-1+2R	72.3	91.9	112.4
8S-GLP-1	97.9	111.4	83.6
8S-GLP-1+2R	56.4	95.5	91.2
8S-GLP-1+3R	−17.0	75.6	101.9
8S-GLP-1+4R	34.0	64.7	105.5
8S-GLP-1+5R	54.3	48.6	75.6
8S-GLP-1+6R	64.9	81.1	80.6
8S-GLP-1+8R	74.5	87.4	95.9
8S,des36R-GLP-1+1KR	100.1	112.9	80.5
8S,des36R-GLP-1+2KR	36.2	88.9	81.6
8S,des36R-GLP-1+3KR	45.0	96.6	86.7
8S,des36R-GLP-1+5KR	7.9	55.3	63.4
8S,des36R-GLP-1+7KR	8.8	55.6	92.6
8S,des36R-GLP-1+10KR	7.5	20.8	55.4
8S-GLP-1+2K	53.2	103.8	88.8

As a result, any GLP-1 derivatives had an in vitro activity of producing cyclic AMP. However, there was a tendency for the activity to be decreased as the number of arginine or lysine

5   residues added was increased. This tendency was recognized particularly with respect to lysine. However, it is highly possible that the added arginine or lysine residues will be cleaved by peptidase upon absorption via a mucous membrane, and thus this in vitro activity does not necessarily reflect the

10 in vivo activity, and this inconsistence may bring about results

in in vivo tests described below.

Test Example 2: Blood glucose lowering activity and insulin
secretion stimulating activity of GLP-1 derivatives absorbed
via mucous membrane

5   An increase in absorption of the GLP-1 derivative via a
mucous membrane was evaluated in terms of in vivo blood glucose
lowering activity and insulin secretion stimulating activity.
That is, the GLP-1 derivative was administered via the nose into
mice, and evaluated in an oral glucose tolerance test (OGTT)
10   where a change in blood glucose level in the mice after tolerated
with glucose was measured.

      The GLP-1 derivative was dissolved in distilled water to
give 1 mM solution which was then stocked at -80°C. At the time
of the test, the solution was diluted to a predetermined
15   concentration with physiological saline.

      Mice were anesthetized lightly with ether. 20 μl GLP-1
derivative solution was taken in a micropipette and discharged
through the tip of the micropipette into the nose of each mouse.
The GLP-1 derivative solution was inhaled by breathing through
20   the nose into the mouse. Five minutes after the nasal
administration of GLP-1 derivative, 5% glucose solution was
orally administered through a probe in an amount of 10 ml/kg.

      The blood glucose level was determined by collecting
several μl blood from a cut in the tail just before the test
25   and 5, 10 and 20 minutes after glucose administration, and

measuring the blood with a small blood-glucose-level measuring
instrument (Glutest Ace, manufactured by Sanwa Kagaku Kenkyusho
Co., Ltd.). The area under the blood concentration-time curve
(AUC 0-20 min) as an increase from the blood glucose level in
5   each mouse before administration of the GLP-1 derivative was
calculated.

       The blood insulin level was determined by collecting 75
μl blood from an orbital venous plexus with a heparin-treated
glass capillary 5 minutes after administration of glucose, then
10   centrifuging the blood and measuring plasma insulin by EIA (Levis
Mouse Insulin Kit, manufactured by Shibayagi Co., Ltd.).

       The blood glucose level and blood insulin level in the
group given GLP-1 derivatives, expressed in terms of average
and standard error, are shown in Table 2.

15

Table 2

Blood glucose level and blood insulin level upon administration of GLP-1 derivative in OGTT test

Administered sample	Dose (nmol/mouse)	Blood glucose level (area under blood concentration-time curve $(AUC_{0-120 min})$ (mg/dl min)			Blood insulin level (5 minutes after glucose administration (ng/mL)		
Physiological saline	0	712	±	103	632	±	107
Naturally occurring GLP-1	1.07	697	±	94	662	±	91
Naturally occurring GLP-1	10.7	399	±	35	743	±	147
8S-GLP-1	1.07	624	±	89	1026	±	199
8S-GLP-1	10.7	291	±	66	1289	±	165
8S-GLP-1+2R	1.07	535	±	58	957	±	86
8S-GLP-1+3R	1.07	509	±	92	1359	±	318
8S-GLP-1+4R	1.07	388	±	54	1713	±	430
8S-GLP-1+5R	1.07	483	±	26	1504	±	250
8S-GLP-1+6R	1.07	559	±	27	1633	±	449
8S-GLP-1+8R	1.07	487	±	32	1882	±	402
8S,des36R-GLP-1+1KR	1.07	611	±	51	1349	±	244
8S,des36R-GLP-1+2KR	1.07	564	±	52	1243	±	309
8S,des36R-GLP-1+3KR	1.07	557	±	53	1176	±	233
8S,des36R-GLP-1+5KR	1.07	404	±	71	2229	±	346
8S,des36R-GLP-1+7KR	1.07	457	±	69	1604	±	344
8S,des36R-GLP-1+10KR	1.07	492	±	106	2379	±	520
8S-GLP-1+2K	1.07	598	±	63	862	±	150

As a result, it was found that GLP-1 derivatives 8S-GLP-1+4R and 8S, des36R-GLP-1+5KR exhibited the strongest blood glucose depressing action. The derivatives having

5　arginine residues added to 8S-GLP-1+4R or more or the derivatives having lysine residues added to 8S, des36R-GLP-1+5KR or more exhibited the strongest insulin secretion stimulating activity. These derivatives in an amount of 1/10 relative to naturally occurring GLP-1 showed a similar effect to that of the naturally

10　occurring one, and thus the absorption of these GLP-1 derivatives was considered to be 10 times as high as that of the naturally occurring GLP-1.

Test Example 3: Action of charge-regulated fat emulsion on

absorption of GLP-1 derivatives via mucous membrane

The blood glucose lowering activity and insulin secretion stimulating activity of GLP-1 derivatives used in combination with the charge-regulated fat emulsion were examined
5   in an oral glucose tolerance test conducted in the same manner as in Text Example 2. The charge-regulated fat emulsion used was prepared according to JP-A 8-27018; that is, a charge-regulated fat emulsion at a final concentration of 8% was obtained by using 2% (w/w) phosphatidyl glycerol (sodium
10   salt), 8% (w/w) neutral oil and 90% (w/w) water.

50 μl of 8% charge-regulated fat emulsion was mixed with 3.56 μl of 1 mM 8S-GLP-1+5R solution and 146.4 μl distilled water to give 2% charge-regulated fat emulsion solution containing 8S-GLP-1+5R at a final concentration of 0.0178 mM. As controls
15   for comparison, 0.534 mM naturally occurring GLP-1 solution (whose concentration is 30 times as high as that of 8S-GLP-1+5R) not containing 2% charge-regulated fat emulsion, 0.0178 mM 8S-GLP-1+5R solution not containing 2% charge-regulated fat emulsion, and physiological saline were used respectively.

20   Mice were anesthetized lightly with ether. 20 μl GLP-1 derivative solution was taken in a micropipette and discharged through the tip of the micropipette into the nose of each mouse. The GLP-1 derivative solution was inhaled by breathing through the nose into the mouse. Five minutes after the nasal
25   administration of GLP-1 derivative, 5% glucose solution was

orally administered through a probe in an amount of 10 ml/kg.

The blood glucose level was determined by collecting several µl blood from a cut in the tail just before the test and 10, 20 and 30 minutes after glucose administration, and

5   measuring glucose with a blood-glucose-level measuring instrument (Glutest Ace, manufactured by Sanwa Kagaku Kenkyusho Co., Ltd.). The area under the blood concentration-time curve (AUC 0-30 min) as an increase from the blood glucose level in each mouse before administration of the GLP-1 derivative was

10 calculated.

The blood insulin level was determined by collecting 75 µl blood from an orbital venous plexus with a heparin-treated glass capillary 10 minutes after administration of glucose, then centrifuging the blood and measuring plasma insulin by EIA

15 (Levis Mouse Insulin Kit, manufactured by Shibayagi Co., Ltd.).

The blood glucose level and blood insulin level in the group given GLP-1 derivatives, expressed in terms of average and standard error, are shown in Table 3.

Table 3

Blood glucose level and blood insulin level in OGTT test upon administration of GLP-1 derivatives used in combination with charge-regulated fat emulsion

Administered sample	Dose (nmol/mouse)	Blood glucose level (area under blood concentration-time curve (AUC$_{0-30 min}$) (ng/dl·min)			Blood insulin level (10 minutes after glucose administration) (ng/mL)		
Physiological saline	0	2122	±	198	251	±	12
Naturally occurring GLP-1	10.7	1533	±	111	377	±	41
8S-GLP-1+5R Used in combination with charge-regulated fat emulsion	0.357	2025	±	176	506	±	34
8S-GLP-1+5R	0.357	1526	±	354	560	±	81

As a result, 8S-GLP-1+5R used in combination with the charge-regulated fat emulsion, in an amount of 1/30 relative to the naturally occurring GLP-1 without charge-regulated fat

5   emulsion, exhibited a similar blood glucose lowering activity similar to that of the naturally occurring GLP-1. That is, the effect of 8S-GLP-1+5R was exhibited at a lower concentration thereof by using the charge-regulated fat emulsion.

Test Example 4: Evaluation of activity of GLP-1 derivative

10   8S26Q34N-GLP-1

The in vitro activity of GLP-1 derivative 8S26Q34N-GLP-1 in producing cyclic AMP was measured according to the method in Test Example 1. After amino acid substitution, the activity was maintained as well (Table 4).

15

Table 4

Cyclic AMP producing activity of 8S26Q34N-GLP-1

Concentration of 8S26Q34N-GLP-1 (log M)	Amount of cAMP produced (pmol/10^5 cell/30min)
-12	0.6
-11	4.7
-10	24.2
-9	76.1
-8	79.2

When the insulin secretion stimulating activity was examined for 30 minutes in the presence of 16.7 mM glucose (high blood glucose condition) by using mouse Langerhans islet, the

5   GLP-1 derivative S26Q34N-GLP-1 showed a stronger insulin secretion stimulating activity than that of the naturally occurring GLP-1 (Table 5).

Table 5

Insulin secretion stimulating activity of 8S26Q34N-GLP-1 in the presence of 16.7 mM glucose by using mouse Langerhans islet

Assay concentration (log M)	Amount of insulin secreted (ng/islet/30min)	
	Naturally occurring GLP-1	8S26Q34N-GLP-1
0	2.61	
-10	4.11	3.64
-9	5.11	6.61
-8	6.71	9.85

The blood glucose lowering activity in mice was examined

10   by administering the GLP-1 derivative subcutaneously into mice, and 0.5 g/kg glucose administered through a tail vain into mice 5 minutes after administration of the GLP-1 derivative. The GLP-1 derivative 8S26Q34N-GLP-1 showed concentration-dependent

depression in blood glucose level, and this activity was stronger

than that of the naturally occurring GLP-1 (Table 6)

Table 6

Activity of 8S26Q34N-GLP-1 derivative on blood glucose level
in mouse

Dose (µg/kg)	Blood sugar level 20 minutes after tolerance with glucose (mg/dl)	
	Naturally occurring GLP-1	8S26Q34N-GLP-1
0	123	
5	119	110
10	103	95
20	75	21

The results of this test indicate that the GLP-1 derivative

5   in Reference Production Example 1 maintains a GLP-1 activity.

When the test results in Table 1 are also taken into consideration,

it can be concluded that even if several residues of arginine

and/or lysine are added to the C-terminus of this GLP-1 derivative,

its GLP-1 activity can be still maintained.

10   Test Example 5: Evaluation of resistance of GLP-1 derivative

8S-GLP-1 to dipeptidyl peptidase IV (DPPIV)

500 pM GLP-1 derivative 8S-GLP-1 was mixed with 40 µU/µl

dipeptidyl peptidase IV and incubated at 37°C for 60 minutes.

Thereafter, the sample was extracted with a 2-fold excess volume

15   of ethanol, centrifuged and evaporated into dryness by an

evaporator.   The resulting dried product was dissolved in

distilled water containing 1% BSA, and its cyclic AMP production

activity was measured according to Test Example 1, to calculate

the residual activity (%).

20   As a result, there was no difference in activity of the

derivative regardless of whether it was treated or not treated with dipeptidyl peptidase IV, thus indicating that this GLP-1 derivative is resistant to dipeptidyl peptidase IV. Accordingly, the GLP-1 derivative can acquire resistance to

5   dipeptidyl peptidase IV by substituting position 8 with serine (Table 7).

Table 7

Peptide	Residual activity (%)	
	DPP IV	
	−	+
8S−GLP−1	100%	101%
Naturally occurring GLP-1	100%	25%

Test Example 6: Evaluation of resistance of GLP-1 derivative 26Q34N-GLP-1 to trypsin

10   The GLP-1 derivative 26Q34N-GLP-1 in Reference Production Example 2 was dissolved at a concentration of 500 µg/ml in 50 mM ammonium bicarbonate at pH 7.8.   Then, 5 µl of 500 µg/ml trypsin (Promega Cat. No. V5113) was added to 100 µl of the solution, and the mixture was incubated at 37°C for 1 hour.   The reaction

15   was terminated by adding 1200 µl of 71.5% (final 65%) ethanol, and the reaction solution was centrifuged at 15,000 rpm at 4°C for 5 minutes to recover a supernatant which was then evaporated into dryness.   The dried product was dissolved in distilled water and measured for cAMP activity by the method in Test Example

20   1, to determine the residual activity (%).

       As a result, there was no difference in activity of the

derivative regardless of whether it was treated or not treated with trypsin, thus indicating that this GLP-1 derivative is resistant to trypsin (Table 8).

Table 8

Peptide	Residual activity (%)	
	Trypsin	
	−	+
26Q 34N−GLP−1	100%	94.8%

5   This result indicates that the GLP-1 derivative is rendered resistant to trypsin by substituting amino acids at positions 26 and 34 in the GLP-1 derivative with glutamine and asparagine, respectively.   Thus, it can be concluded that GLP-1 derivatives having several residues of arginine and/or lysine added to the

10   C-terminus thereof also have resistance to trypsin.

Industrial Applicability

      Clinical application of GLP-1 by subcutaneous injection is advancing at present. This is because GLP-1 is a peptide

15   which cannot be absorbed upon oral administration. The product of the present invention solves this problem and enables administration in a form other than injection. Because treatment of diabetes by using GLP-1 will last for a long time, the treatment without repeated injection is greatly advantageous

20   to the patients.